Renal regenerative medicine

Dankers, Patricia Yvonne Wilhelmina

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INTRARENAL BMP7 DELIVERY FROM A SUPRAMOLECULAR HYDROGEL IN A RAT MODEL OF KIDNEY ISCHEMIA/REPERFUSION INJURY

In chapters 6 and 7 we discussed our recently designed subcapsular intrarenal drug delivery system composed of a supramolecular hydrogel. This system was applied in healthy kidneys showing a minor tissue response and successful delivery of the anti-inflammatory and anti-fibrotic growth factor protein BMP7. In this chapter we investigated whether delivery of BMP7 from this hydrogel is therapeutically effective in short-term, immediate modulation of renal inflammation and extracellular matrix remodelling in a rat model of acute kidney injury induced by uni-lateral ischemia/reperfusion (IR). IR induction was followed by renal subcapsular implantation of placebo and BMP7-loaded hydrogels. The cortical area under the site of implantation was studied after 3 and 7 days. Subcapsular delivery of only 0.30 μg BMP7 from the hydrogels led to a significant reduction in interstitial inflammatory and myofibroblast cell numbers. These findings show that local, intrarenal delivery of an anti-inflammatory and anti-fibrotic drug from a hydrogel carrier is effective in the reduction of acute inflammation and incipient fibrosis.

This chapter has been submitted: Patricia Y.W. Dankers, Marja J.A. van Luyn, Ali Huizinga-van der Vlag, Arjen H. Petersen, Jasper A. Koerts, Anton W. Bosman, Eliane R. Popa, Intrarenal BMP7 delivery from a supramolecular hydrogel reduces inflammation and incipient fibrosis in a kidney disease model of ischemia/reperfusion injury.

8.1 INTRODUCTION

Kidney injury caused by factors such as ischemia/reperfusion, intoxication, high blood pressure and diabetes may lead to irreversible, chronic kidney disease and, ultimately, to kidney failure. Systemic drug delivery[1] can be used to address kidney injury, but has the major disadvantage that high concentrations of drug are needed to accomplish a beneficial effect in the target organ, which are associated with unwanted side effects in other organs. Therefore, the development of local, intrarenal drug delivery systems that display maximal therapeutic efficacy in the organ, and, concomitantly, minimal systemic side effects, is highly desirable.

In order to intervene locally, specifically, and effectively in the acute phase of chronic injury that leads to kidney failure by chronic inflammation and fibrosis[1], we recently proposed a novel drug delivery approach in which a supramolecular[2-4] hydrogel loaded with bone morphogenetic protein 7 (BMP7)[5], was implanted under the renal capsule in healthy kidneys[6-8]. BMP7 was used as a model proteinaceous drug, since it has been characterized with respect to its renal anti-fibrotic capacities.[9] BMP7 antagonizes the action of TGFβ[10], which is the key responsible growth factor for myofibroblasts[11-12] activation and extracellular matrix production in exaggerated renal wound healing[13] and fibrosis[14]. Moreover, it has also been shown that BMP7 has anti-inflammatory properties by inhibiting inflammatory cell infiltration.[15] Others have shown that BMP7 can be delivered intravenously[15] or intraperitoneally[16-17] with good results in animal models of renal injury[14,18].

In the previous studies we showed that a supramolecular hydrogel drug delivery vehicle elicited a minor foreign body response when implanted under the capsule of healthy rat kidneys (Chapter 6). This foreign body response was restricted to the implantation site and had a negligible effect on the renal cortex.[6] BMP7 could be easily incorporated in the supramolecular hydrogel using an organic, solvent-free gelation method that allowed for preservation of the biological activity of the growth factor (Chapter 7). BMP7 was gradually released into the renal interstitium during the degradation of the hydrogel. Importantly, the slight cortical myofibroblast activation that was elicited by the subcapsular implantation of the hydrogel was resolved after local delivery of BMP7.[7] These results support our proposal that this drug delivery system is a promising tool for intrarenal drug administration.

In this chapter, we pursued our previous efforts by delivering a proof-of-concept for the therapeutic effect of our supramolecular hydrogel-based drug delivery system in a rat model of renal ischemia/reperfusion injury (IRI).[19-20] To fully exploit the *in-vivo* degradation behaviour of our hydrogel, we aimed at short-term effects targeting renal inflammation and tissue remodelling. In this first study exploiting intrarenal drug delivery from a subcapsularly implanted hydrogel we showed the beneficial therapeutic effect of local, intrarenal drug delivery of BMP7 in alleviating myofibroblasts formation and inflammation.

8.2 RESULTS

INDUCTION OF ISCHEMIA/REPERFUSION

A model of unilateral ischemia/reperfusion injury (IRI) was used to study the effect of intrarenal drug delivery of BMP7 (Fig. 1). As drug delivery carrier a supramolecular hydrogel of ureido-pyrimidinone (UPy) functionalized poly(ethylene glycol) was developed, as described before (Chapters 6 and 7; Scheme 1).[6-7] In order to obtain BMP7-loaded hydrogels, both the

hydrogelator and lyophilized BMP7 were dissolved in an aqueous solution, after which the two solutions were mixed. This easy formulation procedure allows for fast preparation of the bioactive hydrogels. Before subcapsular implantation of BMP7-loaded and placebo hydrogels, IRI was induced in the left kidney (Fig. 1A,C). Induction of ischemia was confirmed macroscopically by colour change of the kidney (Fig. 1C). After reperfusion of the kidney, the hydrogels were implanted (Fig. 1C). Upon excision of the kidneys at day 3 or day 7, successful induction of IRI was reconfirmed by a colour change of the respective kidneys (Fig. 1D).

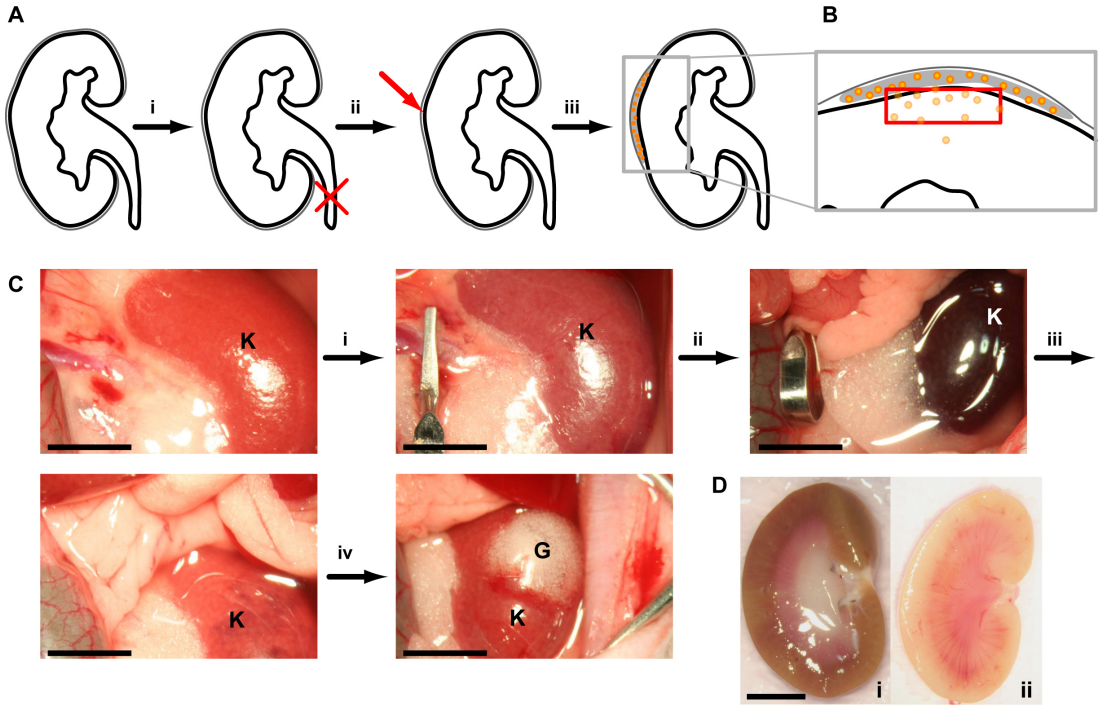
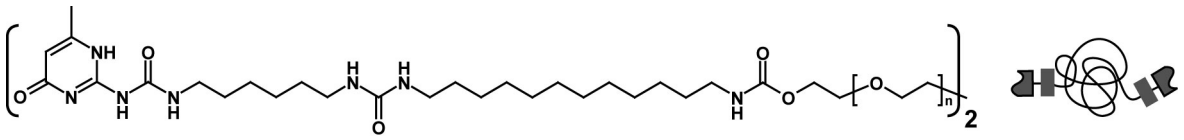


FIGURE 1. SURGICAL PROCEDURES. **A.** Schematic representation of the IRI and implantation procedure: *i.* The renal artery and vena were clamped in the left kidney for 45 min. *ii.* After reperfusion, the renal capsule was loosened from the kidney to introduce a small pocket. *iii.* The hydrogels, without and with BMP7 drug (orange dots), were implanted under the renal capsule. **B.** It is proposed that BMP7 will diffuse into the renal cortex while the hydrogel erodes. The area analyzed for histological examination and morphometry is depicted with a red box. **C.** Space for the clamp was induced. *i.* The renal artery and vena were clamped for 45 min. *ii.* After 45 min of ischemia the kidney had turned dark brown/black. *iii.* Thirty seconds after reperfusion the kidneys recovered their original red colour. *iv.* The hydrogel was implanted under the renal capsule. The kidney **K** and the gel **G** are depicted. **D.** The explanted *i.* contralateral and *ii.* IRI kidneys 7 days after implantation. All scale bars represent 1 cm.



SCHEME 1. CHEMICAL STRUCTURE AND SCHEMATIC REPRESENTATION. The drug delivery carrier is formed after hydrogelation of poly(ethylene glycol), M_n=20 kg/mol, functionalized with two UPy-moieties at the chain ends.

KIDNEY FUNCTION AND GENERAL HISTOLOGY

Subcapsular implantation of placebo hydrogels did not affect kidney function, as previously shown.[6] In the current IRI induction model, no significant differences in creatinine levels were found between treatment groups, indicating no amelioration of the kidney function after intrarenal BMP7 delivery, irrespective of the time points (Fig. 2).

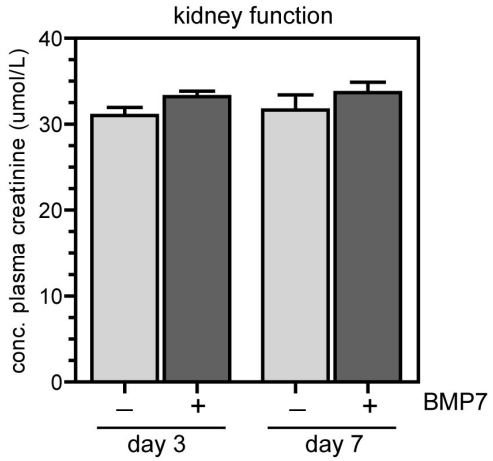


FIGURE 2. BLOOD PLASMA CREATININE CONCENTRATIONS were determined to assess the kidney function, 3 and 7 days after IRI and implantation of the hydrogels without and with BMP7.

As shown before, the foreign body reaction to placebo hydrogel[6], as well as the effect of the BMP7 released[7], is concentrated to the cortical area underneath the implantation site, probably due to its fast degradation profile and release of BMP7 (Chapters 6 and 7).[6-7] Therefore, in this study we investigated the effect of BMP7 in the renal cortex underlying the implantation site by histological examination. General cortical histology in this region was investigated after PAS staining (Fig. 3). As expected, IRI affected the tubular epithelium, while glomeruli were morphologically normal. Cortical tubular damage manifested as epithelial cell flattening, tubular dilatation, tubular denudation and intraluminal casts.[19,21] Histology of BMP7-treated ischemic kidneys, and of ischemic kidneys implanted with placebo hydrogel, was similar at both time points.

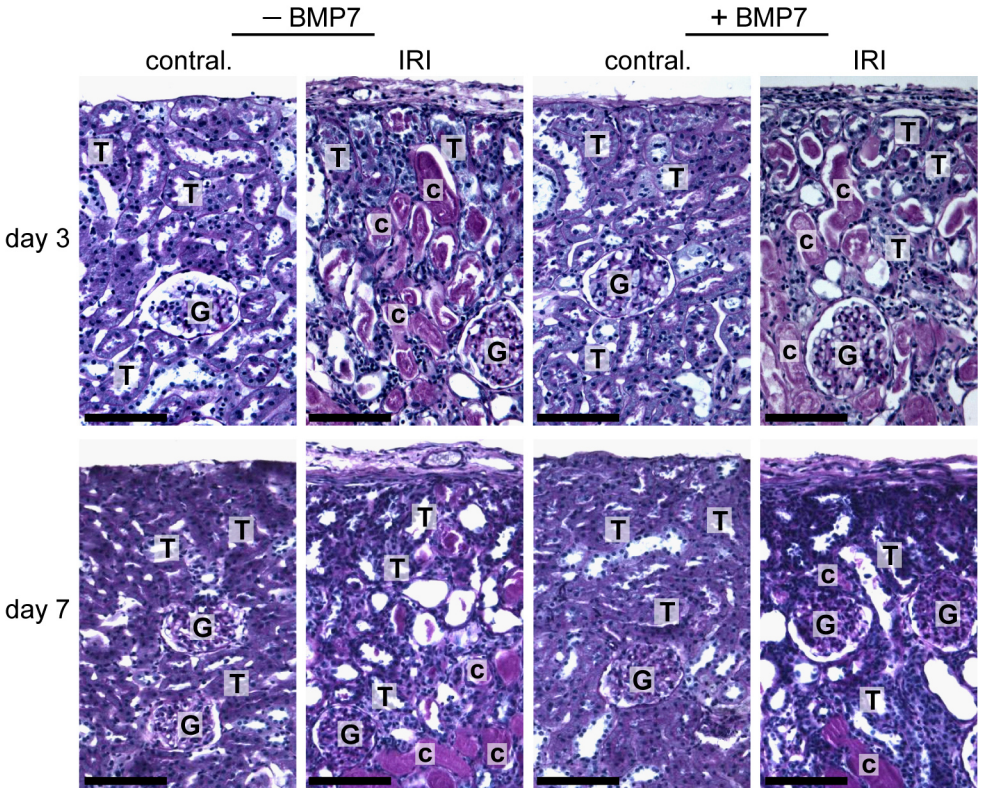


FIGURE 3. THE GENERAL HISTOLOGICAL VIEW OF THE RENAL CORTEX UNDER THE IMPLANTATION SITE, after IRI and implantation of the hydrogels is shown with a PAS staining for the contralateral (contral.) and IRI kidneys without and with BMP7 after 3 and 7 days. All scale bars represent 100 μm. Examples of glomeruli (G), tubules (T) and intraluminal casts (c) are indicated.

PRESENCE OF INTERSTITIAL MYOFIBROBLASTS

The effect of local renal BMP7 delivery on myofibroblast formation, which marks the initial stage of kidney fibrosis, was investigated using an α-smooth muscle actin (αSMA) staining (Fig. 4). In contralateral kidneys, αSMA positive cells were detected sporadically at both time points. After IRI, a strong increase in interstitial myofibroblasts was observed in both treatment groups. However, the number of myofibroblasts in BMP7-treated kidneys was significantly lower than in kidneys without BMP7 at both time points, i.e. a reduction of 5% and 7% was detected at days 3 and 7, respectively (Fig. 4B). In both treatment groups the number of myofibroblasts increased from day 3 to 7, possibly due to the early burst delivery of BMP7 and exhaustion of the growth factor thereafter.

Since myofibroblasts are important producers of extracellular matrix (ECM), we investigated whether the decrease in myofibroblasts after BMP7 delivery was paralleled by a decrease in collagen III deposition (Fig. 5). As expected, collagen III deposition was higher after induction of IRI. However, no differences in collagen III deposition were found in BMP7- and placebo-treated kidneys.

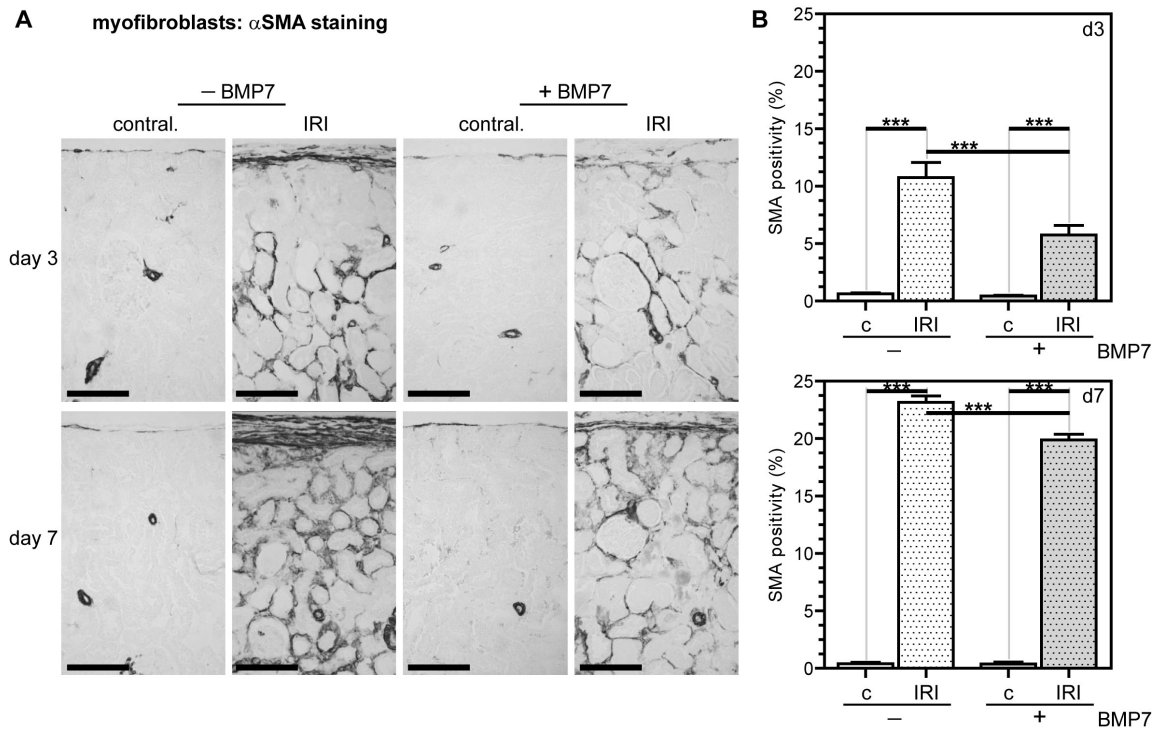


FIGURE 4. THE PRESENCE OF MYOFIBROBLASTS, i.e. αSMA positive cells, was studied in the renal cortex under the implantation site. **A.** Micrographs are shown for the contralateral (contral.) and IRI kidneys without and with BMP7 after 3 and 7 days. All scale bars represent 100 μm. **B.** Quantification of the amount of αSMA as the percentage of the total area was performed in the contralateral (c) and IRI kidneys without and with BMP7 at day 3 and 7.

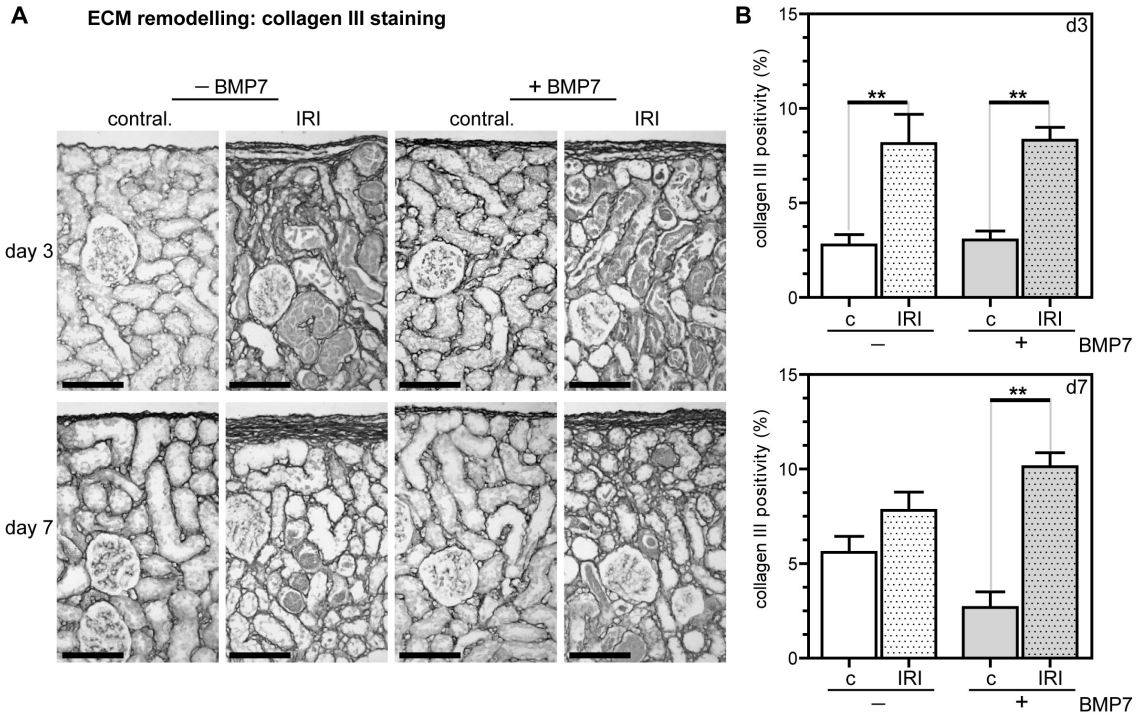


FIGURE 5. THE PRESENCE OF COLLAGEN III was studied in the renal cortex under the implantation site. **A.** Micrographs are shown for the contralateral (contral.) and IRI kidneys without and with BMP7 after 3 and 7 days. All scale bars represent 100 μm. **B.** Quantification of the amount of collagen III as the percentage of the total area was performed in the contralateral (c) and IRI kidneys without and with BMP7 at day 3 and 7.

INTERSTITIAL INFLAMMATION

Besides activation of myofibroblasts and ECM deposition, IRI also triggers inflammation. To determine possible effects of local intrarenal BMP7 delivery on inflammation, cortical interstitial infiltration of bone marrow derived cells (BMDC) was determined using detection of the transgene reporter hPAP (Fig. 6). This approach allows for the intrarenal detection of all BMDC, irrespective of lineage, thus circumventing the possibility that inflammatory cell infiltration is underestimated because of selective staining for specific cell subsets (e.g. macrophage subsets). Few hPAP positive cells were detected in the contralateral kidneys at both time points, reflecting the virtual absence of collateral damage from the ischemic kidneys. In the experimental groups, IRI resulted in interstitial infiltration of BMDC at day 3. However, no differences between treatment groups were detected at this time point. On the contrary, the differences between the experimental groups treated with BMP7 or placebo were clearly present at day 7 (Fig. 6B). The presence of BMP7 stabilized the amount of BMDC, whereas IRI kidneys treated with placebo showed an increase in BMDC of 5.5%.

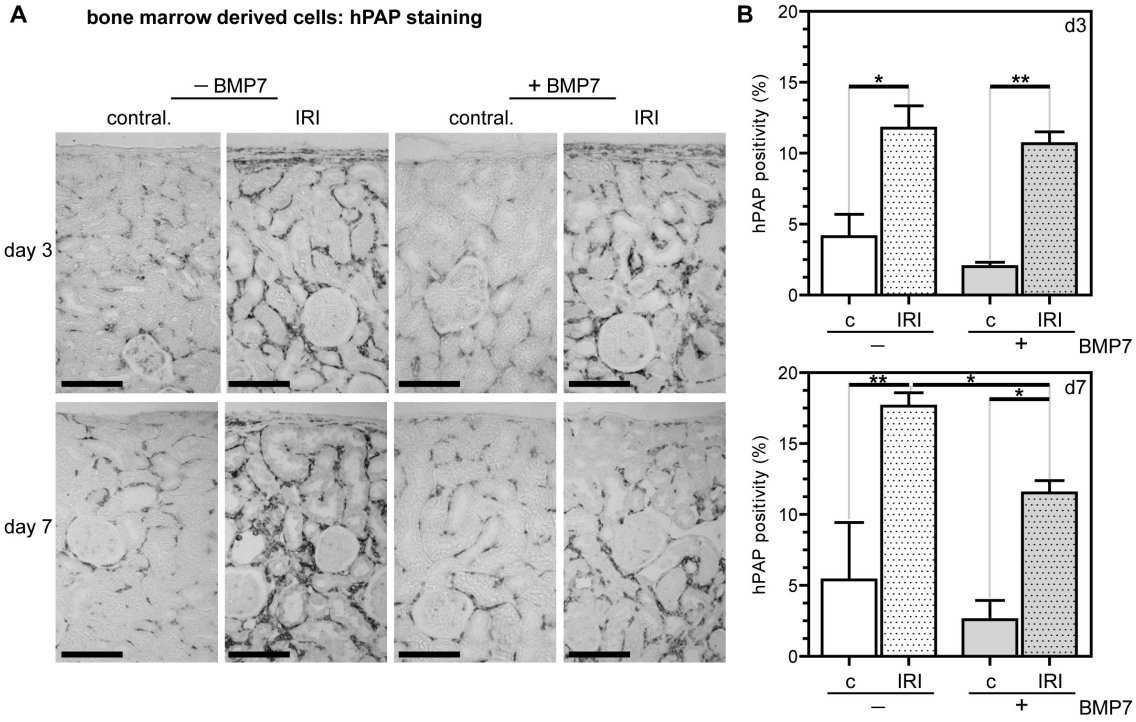


FIGURE 6. THE PRESENCE OF BONE MARROW DERIVED, I.E. HPAP POSITIVE CELLS, was studied in the renal cortex under the implantation site. **A.** Micrographs are shown for the contralateral (contral.) and IRI kidneys without and with BMP7 after 3 and 7 days. All scale bars represent 100 μm. **B.** Quantification of the amount of hPAP as the percentage of the total area was performed in the contralateral (c) and IRI kidneys without and with BMP7 at day 3 and 7.

TUBULAR EPITHELIAL CELL PROLIFERATION AND CALCIUM-SALT DEPOSITION

Besides its anti-fibrotic function, BMP7 has been shown to promote epithelial proliferation and differentiation during nephrogenesis. We therefore investigated the influence of local intrarenal delivery of BMP7 on the induction of tubular epithelial cell proliferation and thus repair. Proliferation of tubular epithelial cells was assessed by nuclear incorporation of BrdU (Fig. 7). Tubular cell proliferation was only studied at day 7 after IRI, because after surgery a recovery period of at least 3 days was required prior to BrdU administration. BrdU

incorporation in tubular epithelial cells was equal in the ischemic kidneys containing placebo hydrogels and in kidneys with the BMP7-loaded hydrogels.

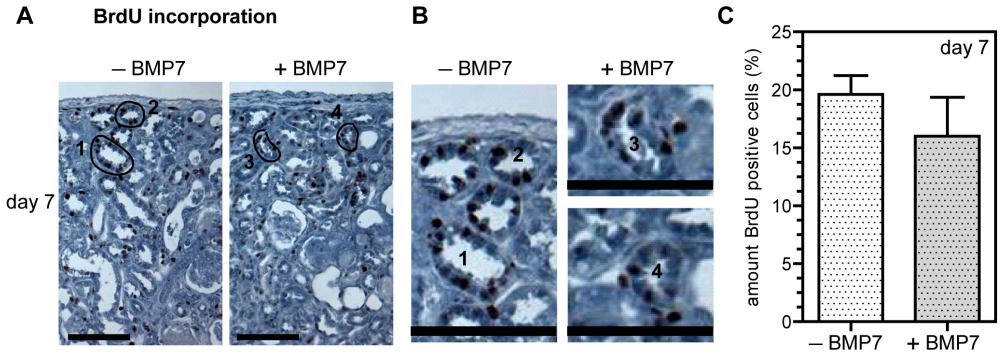


FIGURE 7. CELL PROLIFERATION WAS ASSESSED BY INCORPORATION OF BRDU IN NUCLEI. BrdU-positive cells in the tubuli were quantified in the renal cortex under the implantation site. **A.** Micrographs are shown for the IRI kidneys without and with BMP7 after 7 days. Examples of tubuli containing BrdU-positive and negative cells are highlighted by a circle (and number). **B.** Magnification of the tubuli examples. **C.** Quantification of the number of BrdU-positive cells as the percentage of the total area was performed in the IRI kidneys without and with BMP7 at day 7. All scale bars represent 100 µm.

As BMP7 is known for its osteogenic effects, we assessed whether BMP7 induces osteogenesis in the kidney (as a negative side effect). Calcium-salt deposition was detected by alizarin red staining (Fig. 8). No alizarin red staining was detected in contralateral kidneys at both time points. At day 3, also in the ischemic kidneys no alizarin red signal was detected irrespective of BMP7 treatment (Fig. 8). At day 7, calcium-deposits were observed in the BMP7-treated kidneys, but also in the kidneys that only received the placebo hydrogels. These findings show that BMP7 does not induce osteogenesis in the kidneys studied, but that 7 days after ischemia/reperfusion injury signs of osteogenesis can occur.

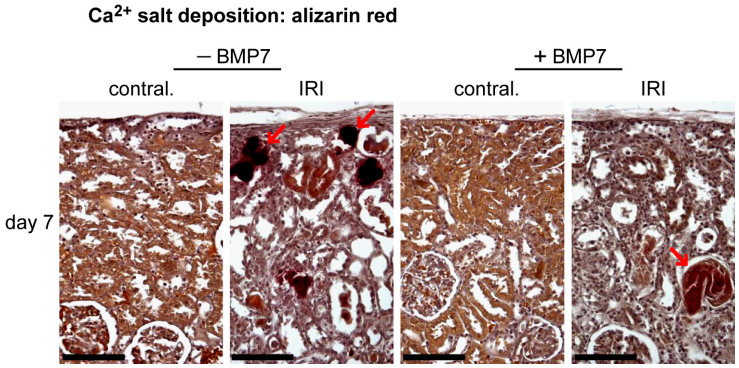


FIGURE 8. THE PRESENCE OF CALCIUM-SALT DEPOSITS was studied in the renal cortex under the implantation site. Micrographs are shown for the contralateral (contral.) and IRI kidneys without and with BMP7 after 7 days. Examples of calcium-salt deposits are indicated with a red arrow. All scale bars represent 100 µm.

8.3 DISCUSSION

In the present chapter we demonstrate the effect of intrarenal, subcapsular delivery of the anti-fibrotic growth factor BMP7 from a supramolecular hydrogel in a rat model of renal ischemia/reperfusion injury. The supramolecular hydrogel is formed in an aqueous environment without the need of an organic solvent. This allows for the easy incorporation of proteinaceous drugs, such as growth factors, which would be denatured in an organic solvent. In addition, this hydrogel is designed for rapid degradation and release within days, aiming for applications in which early intervention in a pathological process, such as inflammation and early tissue remodelling, is desired.

Since BMP7 has been reported to have anti-fibrotic effects in the kidney, we quantified the amount of interstitial myofibroblasts[12] at the implantation site. The significant reduction in myofibroblast numbers during one week after IRI induction demonstrated the effective delivery of BMP7 from the subcapsular hydrogel into the renal interstitium and its biological activity *in vivo*, and confirms our previous finding in healthy kidneys (Chapter 7).[7] Moreover, our findings are in accordance with the results of Hruska *et al.* who applied systemic BMP7 delivery in an unilateral ureteral obstruction (UUO) model.[17] Although myofibroblasts are the major producers of fibrosis-associated collagens[11], such as collagen III, we did not observe a reduction of this extracellular matrix protein after intrarenal BMP7 delivery. This result is in line with our previous finding that peak protein levels for collagen III in this model can only be measured after IRI around day 14.[20] Since our drug delivery system was tuned to early application after damage, we propose that BMP7 delivery from a supramolecular hydrogel with a long term degradation and release profile would allow the study of drug delivery on extracellular matrix production.[6]

Next to its myofibroblast-suppressing effects, renal subcapsular delivery of BMP7 in our IRI model also reduced the number of interstitial bone marrow derived inflammatory cells. Vukicevic *et al.* found comparable results in a rat model of bi-lateral IRI in which a dose of 10-50 µg BMP7 per animal was administered intraveneously.[15] Similarly, Hruska *et al.* found a decline in inflammation in their rat model of UUO after intraperitoneal delivery of 10-60 µg BMP7 per animal.[17] Notably, both models rely on systemic administration of relatively high doses of BMP7, while in our model effects can be observed after local delivery of relatively low amounts, i.e. 0.30 µg BMP7.

During nephrogenesis, BMP7 has been associated with epithelial differentiation and proliferation. We therefore investigated whether local intrarenal BMP7 delivery would promote proliferation of tubular epithelial cells, which are typically affected by IRI. No effect of BMP7 delivery on epithelial proliferation was detected. This finding is possibly due to the dose of BMP7 used, since it is known that, e.g. in nephrogenesis, a low dose of BMP7 induces an increase in nephron number and cell proliferation, while high dose of BMP7 inhibits cell proliferation.[22] Additionally, the general histological view of the cortex at the site of implantation was similar for all IRI kidneys, irrespective of BMP7 delivery. No differences were detected with respect to proximal epithelial flattening and loss, tubular dilatation, tubular denudation and intraluminal debris and protein casts[19,21], indicating that the tubular compartment might not be a target for BMP7. A possible explanation for this might be that BMP7 delivered into the renal interstitium may be unable to target tubular epithelial cells because of the interposed tubular basal membrane, which is frequently thickened after IRI.

It is well-accepted that BMP7 can induce bone formation when delivered from scaffolds placed at endochondral sites.[23-24] Moreover, it has been shown that BMP7 also stimulates osteogenesis at ectopic sites, for example when delivered from subcutaneously implanted poly(lactic-*co*-glycolic acid) nanospheres embedded in fibrous scaffolds of poly(L-lactic acid).[25] An explanation might be that the combination of BMP7 and the presence of a hard scaffold might create an osteogenic environment. Therefore, we assessed whether local intrarenal administration of BMP7 from our delivery system induced calcification or osteogenesis in the cortical area under the implantation site. In our model, BMP7 did not induce osteogenesis. Our current findings add to our previous proposition that the current BMP7-based supramolecular drug delivery system is safe for intrarenal use.[6-7]

As suggested above, an important advantage of our local subcapsular drug delivery system is the use of a low amount of BMP7. Large amounts of BMP7 were required for systemic administration methods, such as intraveneous[15] or intraperitoneal[17] delivery, where doses of approximately 10-50 μg or 10-60 μg BMP7 per animal, respectively, were no exception. Vukicevic *et al.* showed that only 0.27 μg BMP7 reaches the renal cortex after intravenous injection of 50 μg BMP7, which is less than 0.5% of the total amount of BMP7 injected. In our study we applied a total amount of 0.30 μg per animal/kidney with comparable results as found in the other studies of bi-lateral IRI after intraveneous administration[15] and of UUO after intraperitoneal delivery.[16-17] Moreover, these studies did not address possible side effects of systemic BMP7 delivery in other organs, which cannot be excluded. Thus, owing to the low amount of BMP7 required in our model, our approach is not only expected to be favourable with respect to the reduction of side effects in other organs, but also has an economical benefit by reducing treatment costs.

8.4 CONCLUSIONS

Our data indicate that we successfully applied an intrarenal BMP7 delivery system based on a supramolecular hydrogel. Intrarenal delivery of BMP7 led to a reduction of interstitial myofibroblasts and bone-marrow derived inflammatory cells in a rat model of renal ischemia/reperfusion injury. This new approach was developed for early intervention in acute renal damage processes associated with inflammation, tissue remodelling or fibrosis, and may find clinical application in renal transplant patients whose renal grafts develop post-operative ischemia/reperfusion injury.

8.5 EXPERIMENTAL SECTION

CARRIER PREPARATION

Synthesis and extensive characterization of the bifunctional UPy-modified poly(ethylene glycol) hydrogelator with $M_{n,PEG}$ = 20,000 g/mol was performed as described previously (Chapters 6 and 7).[6-7] Hydrogels without and with recombinant human BMP7 (Peprotech) were prepared by first vigorously mixing of the hydrogelator powder and a 0.9% NaCl aqueous solution at 55-65 °C for 1 h. When homogenous solutions/mixtures were obtained, they were cooled to 37 °C under continuous stirring. Subsequently, the water or the BMP7 protein in water (0.1 mg/mL) were added to the stirred solutions, so that 10 w/w% hydrogels were obtained with 0 μg and 0.30 μg (0.01 nmol) BMP7 per 30 μL of gel. The polymeric solutions were removed from the water bath and placed on the bench at 21 °C to set over a 2-4 h period, which resulted in hydrogel formation. The hydrogels were sterilized with UV for 1 h. Then, they were stored at 4 °C or on ice before use at the day of preparation.

ANIMALS

All animal procedures were approved by the committee for care and use of laboratory animals of the University of Groningen, and performed according to governmental and international NIH guidelines on animal experimentation. Male 9 weeks old Fischer rats (20 rats, F344, Harlan) were used as bone marrow recipients. Male and female 11-13 weeks old R26-hPAP rats (5 rats, F344 background, kind gift of E. Sandgren) transgenic for human placental alkaline phosphatase (hPAP) were used as bone marrow (BM) donors.[26] The rats were placed under conventional housing in a temperature-controlled and humidity-controlled room with 12 hours light/dark cycles. They had access to water and standard rat food *ad libitum*.

SURGICAL PROCEDURES

For bone marrow reconstitution, non-transgenic F344 rats were housed in filter top cages, and drinking water was supplemented with 0.35 w/v% neomycine (in house dispensary) for one week before irradiation. Rats underwent total body irradiation at 9 Gy (IBL637, Cesium-137) for bone marrow ablation, and were reconstituted with total hPAP bone marrow; $2.5 \cdot 10^7$ cells/recipient were intraveneously injected (these BM cells were isolated from the R26-hPAP rats by flushing femurs and tibiae with sterile PBS, after which the erythrocytes were lyzed and the BM cell suspension was filtered through a 10 µm cell strainer). After irradiation, rats were housed in filter top cages for 4.5 weeks, and drinking water was supplemented with neomycine for 3 weeks after irradiation. Five weeks after bone marrow transplantation, rats were subjected to unilateral renal ischemia/reperfusion injury (IRI). Surgery was performed under general inhalation anaesthesia consisting of isoflurane (Abbott) and O_2. The abdomen was shaved and disinfected, a ventral midline incision was made, and the left kidney was gently lifted. The left renal artery and vein were clamped for 45 min. After release of the clamp, a small cut was made in the renal capsule, distal to the ureter, and a subcapsular pocket was generated with a blunt needle. The hydrogels without or with BMP7 were injected into this pocket (30 µL hydrogel per kidney). The contralateral kidneys were left untouched. Drinking water was supplemented with 1 mg/mL 5-bromo-2-deoxyuridine (BrdU, Sigma) for 3 days before termination (i.e. for the 7 days experimental group). After 3 and 7 days, under general anesthesia blood was taken from the animals and the blood was heparinized. The kidneys were perfused *in situ* with saline, after which they were excised. The following groups were studied: day 3 without BMP7 (N=5), day 7 without BMP7 (N=3), day 3 with BMP7 (N=5), and day 7 with BMP7 (N=3). Two experimental groups had N=3 because 4 animals deceased during or after surgery. Bone marrow chimerism was determined with flow cytometry: hPAP positivity was determined to be 83.7 ± 4.5% for the BM transplanted F344 rats relative to the R26-hPAP rats (calculated from: $hPAP^+$ BM F344 = 65.9% and $hPAP^+$ BM R26-hPAP = 78.7%).

TISSUE COLLECTION, PROCESSING AND HISTOLOGICAL EXAMINATION

Kidneys were cut into halves in the sagittal plane. One half was fixed in zinc fixative for 18 h (0.1 M Tris-buffer, 3.2 mM calcium acetate, 23 mM zinc acetate, 37 mM zinc chloride, pH 6.5-7; Merck) and embedded in paraffin. The other half was snap-frozen in liquid N_2 and stored at −80 °C. All histological stainings were performed on 5 µm zinc-fixed paraffin-embedded sections, which were deparaffinized before use. After staining, the sections were mounted in permount (Fisher Scientific). Standard washing steps were performed between incubations. Periodic acid-Schiff (PAS) staining was performed to evaluate the general renal morphology (15 min 1% periodic acid, 30 min Schiff's reagent (Merck), 5 min hematoxylin 37 °C, 10 s 70% ethanol with 1% hydrochloric acid). Alizarin staining was performed to investigate whether calcium-salt deposits are present in the kidneys after BMP7 delivery (3 min Alizarin Red S (Fluka) in water pH 4.2; placenta was used as positive control).To determine the effect of subcapsular BMP7 delivery, the kidney area immediately under the implantation site was studied, and was in the order of the red box shown in Fig. 1. Micrographs of tissue slices were taken on a Leica DMLB microscope with Leica DC300 camera and Leica QWin 2.8 software.

KIDNEY FUNCTION

Plasma creatinine concentrations were determined using the enzymatic colorimetric CREA plus assay (Roche).[27]

IMMUNOHISTOCHEMISTRY

Antigen-retrieval was carried out on dewaxed paraffin-embedded sections, either by incubation in 0.1 M Tris-buffer, pH 9.5 at 65 °C for 30 min (for the hPAP staining), or by incubation in 0.1 M Tris-buffer, pH 9.0 at 80 °C for 18 h (for the α smooth muscle actin (αSMA) staining), or by incubation with 0.1% protease (Sigma) for 10 min (for the collagen III staining), or via incubation with 0.7 M HCl at 37 °C for 25 min and subsequently with 0.1 w/v% pepsin (Roche) in 0.35 M HCl at 37 °C for 10 min (for the BrdU staining). Aspecific staining was blocked with the appropriate 2% animal serum for 30 min, with 2% bovine serum albumin for 30 min, or with 5% bovine serum albumin and 0.5% Tween for 15 min (in the case of the BrdU staining). Endogenous peroxidase was blocked with 0.5-1% hydrogen peroxide (Merck) for 30 min, or with 0.5% phenylhydrazine (Fluka) for 30 min in the case of the BrdU staining. Endogenous biotin was blocked with a biotin blocking kit (Dako). Then, the sections were incubated with the following primary antibodies for 1 h, and the appropriate secondary antibodies for 30 min: rabbit anti-hPAP (1:50; Serotec) with secondary antibody goat anti-rabbit IgG1-biotin (1:100; Southern Biotechnology Associates) and additional streptavidin-peroxidase (1:200; Dako) for 30 min; mouse α-smooth muscle actin (1:200; Dako) with secondary

antibody rabbit-anti-mouse-peroxidase (1:100; Dako) and additional swine-anti-rabbit-peroxidase (1:100; Dako) for 30 min; rabbit anti-rat collagen III (1:100; Serotec) with secondary antibody goat-anti-rabbit-peroxidase (1:100; Dako) and additional rabbit-anti-goat-peroxidase (1:100; Dako) for 30 min; mouse anti-BrdU (1:100; Sigma) with secondary antibody goat anti-mouse IgG1-biotin (1:100; Southern Biotechnology Associates) and additional avidin-biotin complex-peroxidase (1:200; Dako) for 30 min. Colour development was performed using 3,3-diaminobenzidine tetrachloride (brown; Sigma), or with 3-amino-9-ethylcarbazole (red; Sigma) in dimethylformamide and 0.5 M acetate-buffer pH 4.9 in the case of the BrdU staining. If necessary, sections were counterstained with haematoxylin (blue; Merck).

QUANTIFICATION

For quantification of effects of BMP7 delivery, the area at the site of implantation was in the order of the red box as shown in Fig. 1 (as was also described before[6]). The amount of positive staining in the kidney cortex adjacent to the capsule in this area was measured. Positive staining of hPAP, αSMA and collagen III were measured using computerized morphometry on a Leica DMLB microscope with Leica DC300 camera and Leica QWin 2.8 software. At least five 200 times magnified areas in each layer were quantified as percentage of positive staining of the total area, for each contralateral and experimental kidney at day 3 and 7. Finally, the values of the two layers were averaged into one value. Vascular expression of αSMA, and vascular and glomerular expression of collagen III were excluded from the measurements. BrdU incorporation was quantified at day 7 by counting BrdU positive and negative nuclei in tubular cells in at least five 400 times magnified areas in two layers at the site of implantation, and is expressed as the percentage of amount of positive nuclei of the total amount of nuclei in the total area for the experimental kidneys without and with BMP7. Interstitial cells were not taken into account.

STATISTICAL ANALYSIS

The differences between the experimental groups with respect to the plasma creatinine concentration were evaluated with the Kruskal-Wallis test followed by Dunns post-hoc test. For the hPAP, αSMA, and collagen III quantification the differences between the groups without and with BMP7 at each time point were analyzed using one-way ANOVA followed by post-hoc Newman-Keuls test. The differences between the groups without and with BMP7 for BrdU were analyzed with an one-tailed Mann-Whitney test. In all cases a 95% confidence interval was used. All data is expressed as mean ± standard error of mean. Probabilities of $P < 0.05$ were considered to be statistically significant; $P < 0.05$ is depicted as *, $P < 0.01$ is shown as **, $P < 0.001$ is indicated as ***.

ACKNOWLEDGEMENTS

We acknowledge Heleen Rienstra and the clinical chemistry laboratory for performing the creatinine measurements, and Martine Broekema, Bert Meijer and Marco Harmsen for useful discussions. This work is supported by SupraPolix, and the Council for Chemical Sciences of The Netherlands Organization for Scientific Research (CW-NWO).

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